REMARKS

Claims 1, 2, 26, and 37-57 constitute the pending claims in the present application.

Applicants have amended claim 1 without prejudice. Claim 1, as amended, is drawn to a method of increasing expression of vascular endothelial growth factor (VEGF) in a subject with ischemic myocardial tissue. Support for the subject matter of Applicants' amendment is found throughout the specification. Exemplary support can be found, for example, in the following passages of the specification: page 87, line 20; Example 4 on pages 101-106; and the paragraph bridging pages 105-106. The claim amendment introduces no new matter.

Applicants have added new claim 58. Support for the subject matter of claim 58 is found throughout the specification. Exemplary support can be found, for example, on page 4, line 16 – page 5, line 16. Applicants note that claim 58 is fully compliant with 35 U.S.C. 112, sixth paragraph.

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the Final Office Action.

1. The Examiner asserts that the Declaration under 37 CFR 1.131 filed on March 23, 2005 is insufficient to overcome the rejection of claims 1-2, 26, and 37-42 under 35 U.S.C. 103(a) based upon Porter et al. (US 6,613,798, 2003). The Examiner argues that "while the declaration sets forth the conception of a genus of agonist and the reduction to practice a specific species, i.e. a polypeptide, the declaration is silent on the specific chemical structure of the other hedgehog agonists, specifically the small organic molecules as described in claim 1" (Office Action, page 2). Applicants traverse but nonetheless note the Examiner's remarks.

2. Claims 1, 2, 26, 37, 38, and 42 remain rejected, and new claims 43-57 are rejected under 35 U.S.C. 103(a) as allegedly unpatentable over Porter et al. (U.S. 6,613,798, 2003) as evidenced by Pettet et al. (Proc. R. Soc. Lond. B 1996; 263: 487-1493) in view of Ferrari et al. (Basic Res. Cardiol. 1995; 90: 52-54). Applicants traverse this rejection and contend that the rejection is moot in light of the amended claims.

The combination of Porter et al., Pettet et al., and Ferrari et al. fail to satisfy the criteria necessary for rendering the claimed invention obvious. Specifically, to establish a *prima facie*

case of obviousness, the following three criteria must be met: (i) there must be some suggestion or motivation to modify the reference or to combine reference teachings, (ii) there must be a reasonable expectation of success of combining the cited references to arrive at the claimed invention, and (iii) the prior art references must teach or suggest each and every limitation of the claimed invention. MPEP 2142-2143.

Ferrari et al. comment on hybernating myocardium and its revascularization but make no mention or suggestion of hedgehog agonists or the treatment of ischemic conditions. Pettet et al. recite that angiogenesis is involved in wound healing. However, Pettet et al. fail to teach or suggest that hedgehog agonists could be useful as angiogenic agents, or that myocardial function following ischemia may be improved by promoting angiogenesis. Porter et al. recite small molecule hedgehog agonists. However, Porter et al. do not teach that hedgehog agonists can be used to promote angiogenesis or to improve myocardial function. Given the disparate teachings of these references, absent the teachings of the instant application, there would have been no motivation to combine these references and there would have been no reasonable expectation of success at arriving at the claimed invention.

Applicants contend that no common link exists between these cited disclosures that would have motivated a person skilled in the art to combine these teachings with a reasonable expectation of success of arriving at the claimed invention. The Examiner contends that the common link among the three documents is scarring. According to the Examiner, Porter et al. recite a small organic hedgehog agonist that is capable of promoting wound healing following surgery, wherein the wound heals with less scarring, Pettet et al. teach that angiogenesis occurs during wound healing, and Ferrari et al. teach that hybernating myocardium is identified by scar formation following myocardial infarction and that myocardial infarction can be improved after revascularization (pages 6-7 of the Final Office Action).

Applicants argue that scarring as a common link is insufficient to arrive at the claimed invention. Wound healing is a complex process involving many different stages including the inflammatory phase, the proliferative phase, and the maturation and remodeling phase. The inflammatory phase in turn involves platelets and the clotting cascade, vasoconstriction followed by vasodilation, and the cleansing activity of polymorphonuclear neutrophils and macrophages aided by T helper cells. The proliferative phase includes angiogenesis, deposition of collagen by fibroblasts and granulation tissue formation, epitheliazation, and contraction. Accordingly, in

the complex process of wound healing and scarring, angiogenesis is one of many different activities that takes place. The mere reference to the complex process of scarring provides no guidance to one of skill in the art as to whether and how the cited references might interrelate.

Although Pettet et al. may teach that angiogenesis occurs during wound healing, there is no reason to assume that a hedgehog agonist, which can promote wound healing according to Porter et al., promotes wound healing **via angiogenesis**. For example, based on the teachings of Pettet et al. and Porter et al., it would be possible that a hedgehog agonist promotes wound healing by promoting collagen deposition, or by promoting inflammation, or by promoting any other process involved in wound healing that is not angiogenesis. The Examiner states, " Pettet et al. teaches that angiogenesis, the formation of blood vessels, is described as a process whereby capillary sprouts are formed in response to externally supplied chemical stimuli and occurs during wound healing; and therefore, *is inherently involved in Porter et al.'s method of promoting would healing* resulting from surgery..." (Office Action, page 6, *emphasis added*). As a first point, regardless of whether angiogenesis is involved in wound healing, that does not provide any guidance to suggest that hedgehog agonism promotes angiogenesis. For example, based on the cited documents, it would have been equally likely that hedgehog agonism promoted other phases of wound healing, such as epithelial cell proliferation. Porter et al. recite

> Wound repair includes the stages of hemostasis, inflammation, proliferation, and remodeling. The proliferative stage involves multiplication of fibroblasts and endothelial and epithelial cells. *Through the use of the subject method, the rate of proliferation of epithelial cells in and proximal to the wound can be controlled in order to accelerate closure of the wound and/or minimize the formation of scar tissue.* (column 61, lines 39-46, *emphasis added*)

Porter et al. teach that hedgehog agonists can be used to control the growth of epithelial cells. The citation above supports Applicants' contention that a reference to wound healing in Porter et al. does not necessarily teach or suggest a method for promoting angiogenesis. Accordingly, Applicants contend that the mere recitation of scarring or wound healing in the cited references fails to provide either a motivation to combine the cited reference or a reasonable expectation of success at combining the cited referenced to arrive at the claimed invention.

In response to Applicants' previous amendment reciting increased expression of vascular endothelial growth factor (VEGF), the Examiner states that "the claimed limitation does not appear to result in a manipulative difference between the prior arts disclosure of an effective

amount to produce a therapeutic effect, e.g., promotion of wound healing, being .0001 to about 100 mg per kilogram (column 67, lines 46-59) and the claimed limitation" (Final Office Action, page 7). Although the Examiner concedes that Porter et al. do not explicitly teach an amount of a hedgehog agonist effective to promote angiogenesis via increased expression of VEGF, the Examiner asserts that VEGF expression is "inherent". Applicants disagree and contend that VEGF expression does not necessarily and invariably occur during angiogenesis. For example, angiogenesis may be promoted by chemokines (Belperio et al. 2000 J Leukocyte Biol 68: 1-8, enclosed as Exhibit 1) and may be VEGF-independent (Douglas and Nicolls, J Clinical Investigation 2005 115: 1133-1136, first column, second paragraph of page 1135, enclosed as Exhibit 2). The observation that hedgehog induces expression of VEGF is not an inherent feature of hedgehog-promoted angiogenesis, as 1) angiogenesis does not inevitably entail increased expression of VEGF, and 2) the skilled artisan would not have recognized increased VEGF expression (which the Examiner contends is an inherent feature of hedgehog-induced angiogenesis) as a necessary feature of hedgehog-promoted angiogenesis.

Furthermore, the inherent features of wound healing or angiogenesis are insufficient to support a rejection based on obviousness. "That which may be inherent is not necessarily known. Obviousness cannot be predicated on what is unknown." *Application of Spormann*, 53 C.C.P.A. 1375, 363 F.2d 444, 448, 150 U.S.P.Q. (BNA) 449, 452 (1966) (citing *In re Adams*, 356 F.2d 998, 53 CCPA). Given that the present rejection under 35 U.S.C. 103 is predicated on an alleged inherent relationship between VEGF and angiogenesis and between VEGF and hedgehog, and given that the rejection cannot stand absent reliance on these alleged inherent relationships, Applicants respectfully submit that the rejection should be withdrawn.

Nevertheless, and solely to expedite prosecution, Applicants have amended the claims to more particularly recite a method of increasing expression of VEGF in a subject with ischemic myocardial tissue. Applicants' amendments are not in acquiescence to the rejection. Applicants reserve the right to prosecute claims of similar or differing scope.

The combined teaches of the cited references fail to teach or suggest each and every limitation of the claimed method. As detailed above, any argument that the cited references (i) teach or suggest the claimed invention, (ii) provide motivation to combine the cited references, or (iii) offer a reasonable expectation of successfully combining the references to arrive at the claimed invention is based solely on reliance upon the alleged inherent features of angiogenesis,

wound healing, or VEGF expression. Given that the present rejection under 35 U.S.C. 103 is predicated on alleged inherent features of VEGF expression, wound healing, and angiogenesis, and given that the rejection cannot stand absent reliance on these inherent features, Applicants respectfully submit that the rejection should be withdrawn.

3. Claims 39-41 remain rejected under 35 U.S.C. 103(a) as allegedly unpatentable over the aforementioned documents in further view of Igo et al. (US 5,681,278, 1997). Igo et al. teach administration of an agent intracoronarally. However, Igo et al. fail to overcome the deficiencies of the combined teachings of Porter et al., Pettet et al., and Ferrari et al. If an independent claim, for example independent claim 1, is nonobvious under 35 U.S.C. 103, then any claim depending therefrom (e.g., claims 39-41) is nonobvious. *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988). Reconsideration and withdrawal of this rejection is requested.

4. Claims 1, 2, 26, 37, 38, and 42-57 are rejected under 35 U.S.C. 103(a) as allegedly unpatentable over Baron et al. (WO 98/35020, 1998) in view of Porter et al. (US 6,613,798, 2003) as evidenced by Pettet et al. (Proc. R. Soc. Lond. B 1996; 263: 1487-1493). Applicants traverse this rejection and contend that the rejection is moot in light of the amended claims.

The Examiner argues that Baron et al. teach a method of treating a subject suffering from an ischemia in tissues containing mesodermally derived cells comprising administering a compound to the ischemic site so as to stimulate vascular growth, wherein the ischemia is myocardial ischemia and the compound is an agonist of a hedgehog-protein-receptor. However, the claims as amended are drawn to a method of increasing VEGF expression. None of Baron et al., Porter et al., or Pettet et al. teach or suggest that an agonist of the hedgehog pathway can induce expression of VEGF. Furthermore, and as detailed above, any rejection based on the alleged inherent expression of VEGF or an alleged inherent relationship between VEGF and hedgehog is insufficient to support a rejection under 35 U.S.C. 103(a).

Given that the combined teachings of these documents fail to teach or suggest each and every element of the claimed invention, the aforementioned references fail to render obvious the claimed invention. Applicants' amendments are not in acquiescence to the rejection. Applicants reserve the right to prosecute claims of similar or differing scope. In light of Applicants' amendments and arguments, reconsideration and withdrawal of this rejection is requested.

5. Claims 39-41 remain rejected under 35 U.S.C. 103(a) as allegedly unpatentable over Baron et al. (WO 98/35020, 1998) in view of Porter et al. (US 6,613,798, 2003), as evidenced by Pettet et al. (Proc. R. Soc. Lond. B 1996; 263: 1487-1493) in further view of Igo et al. (US 5,681,278, 1997). The Examiner states that Igo et al. teach a method for treating blood vessels in a mammal via intrapericardial injection. Applicants traverse.

As stated above, claim 1 as amended, and all claims that depend from claim 1, are now drawn to a method of increasing expression of VEGF. Igo et al. fail to overcome the deficiencies of Baron et al., Porter et al., and Pettet et al., and thus, the combination of Igo et al. and the other cited documents fail to teach or suggest the claimed invention. If an independent claim, for example independent claim 1, is nonobvious under 35 U.S.C. 103, then any claim depending therefrom (e.g., claims 39-41) is nonobvious. *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988). Reconsideration and withdrawal of this rejection is requested.

CONCLUSION

In view of at least the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945 under Order No. CIBT-P01-119.**

Respectfully Submitted,

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CXC chemokines in angiogenesis

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Abstract: A variety of factors have been identified that regulate angiogenesis, including the CXC chemokine family. The CXC chemokines are a unique family of cytokines for their ability to behave in a disparate manner in the regulation of angiogenesis. CXC chemokines have four highly conserved cysteine amino acid residues, with the first two cysteine amino acid residues separated by one nonconserved amino acid residue (i.e., CXC). A second structural domain within this family determines their angiogenic potential. The NH_2 terminus of the majority of the CXC chemokines contains three amino acid residues (Glu-Leu-Arg: the ELR motif), which precedes the first cysteine amino acid residue of the primary structure of these cytokines. Members that contain the ELR motif (ELR^+) are potent promoters of angiogenesis. In contrast, members that are inducible by interferons and lack the ELR motif (ELR^-) are potent inhibitors of angiogenesis. This difference in angiogenic activity may impact on the pathogenesis of a variety of disorders. *J. Leukoc. Biol.* 68: 1–8; 2000.

Key Words: cytokines · neovascularization · wound repair · tumorigenesis · tumor metastasis

INTRODUCTION

Angiogenesis is the formation of new blood vessels from preexisting microvasculature. Angiogenesis is a biological process that is critical to both physiological and pathological processes [1–11]. The regulation of angiogenesis depends on a dual, yet opposing balance of angiogenic and angiostatic factors that promote or inhibit neovascularization, respectively. For example, under homeostatic conditions the rate of normal capillary endothelial cell turnover is measured in months or years [12, 13], suggesting a balance in the biological effect of angiogenic and angiostatic factors. During wound repair the formation of granulation tissue is associated with a shift in the balance favoring the predominance of angiogenic factors that supports the development of new functioning capillaries within days [8]. In contrast, the latter phases of wound repair are associated with a marked decline of angiogenesis. This event correlates with the involution of granulation tissue that is concomitant with re-epithelialization. These events suggest that angiogenesis of wound repair is tightly controlled and temporally related to the imbalance of expression of angiogenic and angiostatic factors that ultimately regulates angiogenesis. The temporal imbalance in angiogenic and angiostatic factors in granulation tissue can either represent a marked reduction in the elaboration of angiogenic factors and/or a simultaneous increase in factors that inhibit neovascularization [14]. In contrast to the precise regulation of angiogenesis in wound repair, aberrant angiogenesis can lead to an imbalance in the relationship of angiogenic and angiostatic factors that favors persistent angiogenesis. This type of environment can contribute to the pathogenesis of tumor growth and metastases, and the promotion of chronic fibroproliferative disorders. The complement of angiogenic and angiostatic factors may vary among different physiological and pathological settings. However, the recognition of this dual mechanism of control is critical in order to gain insight into this complex process and understand aberrant angiogenesis associated with a variety of pathological conditions. The CXC chemokines are a unique family of cytokines that can regulate angiogenesis in a disparate manner and will be the subject of this review.

CXC CHEMOKINES, CXC CHEMOKINE RECEPTORS, AND ANGIOGENESIS

CXC chemokines are characteristically heparin binding proteins. On a structural level, they have four highly conserved cysteine amino acid residues, with the first two cysteines separated by one nonconserved amino acid residue [15–27]. Although the CXC motif distinguishes this family from other chemokine families, a second structural domain dictates their angiogenic activity. The NH_2 terminus of the majority of the CXC chemokines contain a three-amino-acid motif (Glu-Leu-Arg: the ELR motif), which precedes the first cysteine amino acid of the primary structure of these cytokines [15–27]. The family members that contain the ELR motif (ELR^+) are potent promoters of angiogenesis [23] (**Table 1**). In contrast, members

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Exhibit 1

TABLE 1. ELR$^+$ and ELR$^-$ CXC Chemokines Are Angiogenic and Angiostatic Factors, Respectively

Angiogenic CXC chemokines containing the ELR motif (ELR$^+$)
Interleukin-8 (IL-8)
Epithelial neutrophil activating protein-78 (ENA-78)
Growth-related gene alpha (GRO-α)
Growth-related gene beta (GRO-β)
Growth-related gene gamma (GRO-γ)
Granulocyte chemotactic protein-2 (GCP-2)
Platelet basic protein (PBP)
Connective tissue activating protein-III (CTAP-III)
Beta-thromboglobulin (β-TG)
Neutrophil activating protein-2 (NAP-2)
Angiostatic CXC chemokines that lack the ELR motif (ELR$^-$)
Platelet factor-4 (PF4)
Interferon-γ-inducible protein (IP-10)
Monokine induced by interferon-γ (MIG)

that are induced by interferons and lack the ELR motif (ELR$^-$) are potent inhibitors of angiogenesis [23, 28–30] (Table 1). Therefore, on a structural/functional level, members of the CXC chemokine family can either promote or inhibit angiogenesis, and the imbalance of the local expression of these chemokines may be important in the regulation of angiogenesis under both physiological and pathological conditions.

Angiogenic (ELR$^+$) CXC chemokines

Members of the CXC chemokine family that behave as angiogenic factors include interleukin-8 (IL-8), epithelial neutrophil activating protein-78 (ENA-78), growth-related genes (GRO-α, -β, and -γ), granulocyte chemotactic protein-2 (GCP-2), and NH_2-terminal truncated forms of platelet basic protein (PBP), which include connective tissue activating protein-III (CTAP-III), beta-thromboglobulin (β-TG), and neutrophil activating protein-2 (NAP-2) [23, 31–33] (Table 1). ELR$^+$ CXC chemokines directly induce endothelial cell chemotactic and proliferative activity *in vitro*, and angiogenesis *in vivo* in the absence of preceding inflammation [23, 32–35]. Their angiogenic activity is distinct from their ability to induce inflammation.

Although a specific CXC chemokine receptor(s) that mediates the angiogenic activity of these cytokines remains to be determined, the candidate CXC chemokine receptors for this effect are CXCR1 and/or CXCR2. Only IL-8 and GCP-2 specifically bind to CXCR1, whereas all ELR$^+$ CXC chemokines bind to CXCR2 [15–27]. The ability of ELR$^+$ CXC chemokine ligands to bind to CXCR2 supports the notion that this represents the receptor for the mediation of angiogenic activity by ELR$^+$ CXC chemokines. This is further supported by the fact that CXCR2 has the greatest sequence homology with the recently described human Kaposi's sarcoma herpes virus-G protein-coupled receptor (KSHV-GPCR; ORF 74) [36–40].

The KSHV-GPCR demonstrates constitutive activation with the ability to cause oncogenic transformation of NIH 3T3 cells and to promote angiogenesis *in vivo* [36, 40]. Infection of primary endothelial cells with KSHV and expression of KSHV-GPCR leads to enhanced proliferation and long-term survival [38]. The CXC chemokine ligands, IL-8 and GRO-α, can act as agonists for KSHV-GPCR, and further augment the signaling of this receptor [41, 42]. It is interesting that introducing a single point mutation in wild-type CXCR2 and transfection of NIH 3T3 cells results in oncogenic transformation in a similar manner as KSHV-GPCR [36]. Moreover, wild-type CXCR2 expression in these cells results in cellular transformation related to autocrine stimulation by an ELR$^+$ CXC chemokine [36]. However, oncogenic transformation was not induced with either mutated CXCR1 or wild-type CXCR1 [36]. These data suggest that either constitutive activation or persistent autocrine stimulation of CXCR2 causes cellular transformation similar to KSHV-GPCR. Thus, the potential expression of CXCR2 on endothelial cells in the presence of persistent autocrine (endothelium), juxtacrine (endothelium), and paracrine (tumor cells or other activated stromal cells) stimulation with ELR? CXC chemokines has important implications in promoting angiogenesis. Future studies will delineate whether CXCR2 is the putative receptor for mediating ELR$^+$ CXC chemokine angiogenic activity.

Angiostatic (ELR$^-$) CXC chemokines

The angiostatic members of the CXC chemokine family include PF4, monokine induced by interferon-γ (MIG), and interferon-γ-inducible protein (IP-10) [43–48] (Table 1). Although stromal cell-derived factor (SDF-1) is another ELR$^-$ CXC chemokine, it remains unclear whether this ELR$^-$ CXC chemokine inhibits or promotes angiogenesis. SDF-1 has been found to induce *in vitro* migration of human umbilical vein endothelial cells [49, 50]. Mice with targeted disruption of the SDF-1 gene perinatally die [51]. This appears to be multi-factorial and includes defects in B cell and myeloid progenitors, suggesting that SDF-1 is involved in lymphopoiesis and myelopoiesis. In addition, these mice demonstrate cardiac ventricular septal defects [51]. Recently, targeted disruption of the receptor for SDF-1, CXCR4, has demonstrated that this CXC chemokine receptor is essential for vascularization of the gastrointestinal tract, hematopoiesis, and cerebellar development in these mice [52, 53]. In contrast to these findings, SDF-1 can attenuate the angiogenic activity of ELR$^+$ CXC chemokines, bFGF, or VEGF [54]. Thus, the role of SDF-1 in modulating angiogenesis in the context of tumorigenesis or chronic fibroproliferative disorders awaits further study.

All three interferons (IFN-α, -β, and -γ) stimulate the expression of IP-10 [15, 43–48]. MIG is induced only by IFN-γ [15, 43–48]. Recently, a new ELR$^-$ member of the CXC chemokine family, IFN-inducible T cell alpha chemoattractant (I-TAC), has been cloned, and is induced primarily by IFN-γ [55]. I-TAC, similar to IP-10 and MIG, inhibits neovascularization in the rat corneal micropocket (CMP) assay of angiogenesis in response to either ELR$^+$ CXC chemokines or VEGF (unpublished observation). These findings suggest that all interferon-inducible ELR$^-$ CXC chemokines are potent inhibitors of angiogenesis. Moreover, this interrelationship of interferon and interferon-inducible ELR$^-$ CXC chemokines and their biological function are directly relevant to the function of IL-18 and IL-12. The capability of IL-18 and IL-12 to induce IFN-γ and subsequent interferon-inducible ELR$^-$ CXC chemokines explains their ability to inhibit angiogenesis [56]. Therefore, IL-12 and IL-18, via the induction of IFN-γ, will have a profound effect on the production of IP-10, MIG, and

I-TAC. The subsequent expression of interferon-inducible ELR^- CXC chemokines may represent the final common pathway and explain the mechanism for the attenuation of angiogenesis related to interferons. Although all three IFN-inducible ELR^- CXC chemokines specifically bind to the CXC chemokine receptor, CXCR3 [55, 57], and the expression of CXCR3 mRNA has been associated with endothelial cells [58], it remains to be determined whether CXCR3 is the putative receptor for interferon-inducible ELR^- CXC chemokine inhibition of angiogenesis.

Recently, eloquent studies have delineated potential mechanisms for the ELR^- CXC chemokine, PF4, and its ability to inhibit angiogenesis that may be relevant to interferon-inducible ELR^- CXC chemokines. The ability of PF4 to bind to glycosaminoglycans (GAG; heparin and heparan sulfate) with high affinity appears to be important to several of its biological functions. PF4 inhibits endothelial cell migration, proliferation, and *in vivo* angiogenesis in response to bFGF or VEGF [59, 60]. Moreover, fluorescein isothiocyanate (FITC)-labeled PF4 injected systemically, selectively binds to the endothelium only in areas of active angiogenesis [61, 62]. This suggests that the microvasculature is the major target for the biological effects of PF4 during angiogenesis. PF4 has been shown to inhibit bFGF and $VEGF_{165}$ binding to their respective receptors [63–65]. One mechanism for this effect is related to the generation of PF4-bFGF or PF4-$VEGF_{165}$ heterodimeric complexes, which impairs bFGF or $VEGF_{165}$ binding to their respective receptors [64–66]. bFGF must undergo dimerization in the presence of endogenous heparin in order to bind to its receptor [65, 66]. PF4 complexes to bFGF and prevents bFGF dimerization followed by impaired receptor binding and internalization [65]. $VEGF_{165}$ possesses heparin binding ability similar to bFGF. PF4 impairs $VEGF_{165}$ binding to its receptors on endothelium via a mechanism similar to what has been reported for its ability to inhibit bFGF [64]. Although the ability of PF4 to form heterodimers with bFGF and $VEGF_{165}$ is one potential mechanism to inhibit bFGF and $VEGF_{165}$ biological activity, it appears that PF4 may inhibit angiogenesis through additional mechanisms.

Although PF4 inhibits specific $VEGF_{165}$ binding, it does not inhibit $VEGF_{121}$ binding to VEGF receptors on endothelial cells. In contrast to $VEGF_{165}$, $VEGF_{121}$ is not a heparin-binding protein [64, 67, 68]. PF4 neither forms heterodimers with $VEGF_{121}$ nor competitively interferes with $VEGF_{121}$ binding to its receptor. However, PF4 directly inhibits $VEGF_{121}$-induced endothelial cell proliferation [64]. These findings suggest that other mechanisms must be operative for PF4 inhibition of mitogen stimulation of endothelial cells, perhaps mediated through its own independent biological signal. Although a specific receptor for PF4 on endothelium has not yet been discovered, studies have suggested that PF4 inhibits endothelial cell cycle by preventing cell entry into S phase [60]. In a model system of endothelial cell stimulation independent of interaction with cell-surface GAGs, PF4 inhibits epidermal growth factor (EGF)-stimulated endothelial cell proliferation by causing a decrease in cyclin E-cyclin-dependent kinase 2 (cdk2) activity that results in attenuation of retinoblastoma protein (pRb) phosphorylation [69]. The mechanism is related to PF4-dependent sustained increase in the levels and binding of the cyclin-dependent kinase inhibitor (CKI), $p21^{Cip1/WAF1}$, to the cyclin E-cdk2 complex. This inhibits cell cycle progression by preventing the down-regulation of $p21^{Cip1/WAF1}$ leading to inhibition of both cyclin E-cdk2 activity and phosphorylation of pRb [69]. These studies suggest that PF4 can inhibit a variety of endothelial cell mitogens at multiple levels. These events may be relevant to interferon-inducible ELR^- CXC chemokines because IP-10 has been shown to compete with PF4 for binding, and inhibition of endothelial cell proliferation that may be related to inhibition of the cell cycle [29]. This supports the notion that interferon-inducible ELR^- CXC chemokines may have similar mechanisms for their inhibition of bFGF, VEGF, EGF, and ELR^+ CXC chemokine-induced angiogenesis.

ELR^+ CXC chemokines promote angiogenesis associated with tumorigenesis

The ELR^+ CXC chemokines are important mediators of tumorigenesis related to their angiogenic properties. Although GRO-β has been recently reported to inhibit angiogenesis [70], the concentration used in this study was 1000-fold higher (1–10 μM) than what was found for its angiogenic activity (1–10 nM) [23, 54]. This would suggest that superphysiological concentrations of GRO-β can desensitize the angiogenic response. Moreover, studies in melanoma tumors support that all GROs play a significant role in mediating tumorigenesis related to both their mitogenic and angiogenic activities. For example, GRO-α, -β, and -γ have all been found to be highly expressed in human melanoma [71]. To determine the biological significance of the presence of these ELR^+ CXC chemokines in melanoma, human GRO-α, -β, and -γ genes have been transfected into immortalized murine melanocytes [71, 72]. The persistent expression of GROs in these cells transforms their phenotype to one with anchorage-independent growth *in vitro* and the ability to form tumors *in vivo* in nude and SCID mice [71, 72]. The tumors are highly vascular and similar to the vascularity of B16 melanoma controls [71, 72]. When tumors are depleted of GROs there is a marked reduction of tumor-derived angiogenesis directly related to inhibition of tumor growth [71, 72]. These findings support the notion that the ELR^+ CXC chemokines, such as GRO-α, -β, and -γ, have the ability to act both as autocrine growth factors for melanoma and as potent paracrine mediators of angiogenesis to promote tumorigenesis and metastases.

The progression and growth of ovarian carcinoma is also dependent on successful angiogenesis, and IL-8 has been determined to play a significant role in mediating human ovarian carcinoma-derived angiogenesis and tumorigenesis [73]. The expression of IL-8, bFGF, and VEGF was examined in five different human ovarian carcinoma cell lines [73]. All cell lines *in vitro* expressed similar levels of bFGF, however, these cells expressed either high or low levels of IL-8 or VEGF. When implanted into the peritoneum of nude mice, the high-expressing IL-8 tumors were associated with all animals dying in <51 days [73]. The expression of IL-8 was directly correlated with neovascularization and inversely correlated with survival, whereas VEGF expression was only correlated with production of ascites [73]. No correlation was found for bFGF with either tumor neovascularization or survival [73]. This

study has been substantiated in patients with ovarian cancer, where ascites fluid demonstrates angiogenic activity directly correlated to IL-8 [74]. These findings support the notion that antigenic ELR$^+$ CXC chemokines play a greater role than bFGF and VEGF in mediating angiogenesis associated with ovarian cancer.

IL-8 is markedly elevated and contributes to overall angiogenic activity of non-small-cell lung cancer (NSCLC) [75]. Extending these studies to an *in vivo* model system of human tumorigenesis (i.e., human NSCLC/SCID mouse chimera) [76], tumor-derived IL-8 was found to be directly correlated with tumorigenesis [76]. Tumor-bearing animals depleted of IL-8 demonstrated a >40% reduction in tumor growth and a reduction in spontaneous metastases [76]. The attenuation of tumor growth and metastases was directly correlated to reduced angiogenesis. These findings have been further corroborated through the use of several human NSCLC cell lines grown in nude mice. NSCLC cell lines that constitutively express IL-8 display greater tumorigenicity that is directly correlated to angiogenesis [77].

Although IL-8 was the first angiogenic CXC chemokine to be discovered in NSCLC, ENA-78 was found to be highly correlated with NSCLC-derived angiogenesis [78]. Surgical specimens of NSCLC tumors demonstrate a direct correlation of ENA-78 with tumor angiogenesis. These studies were extended to a SCID mouse model of human NSCLC tumorigenesis. ENA-78 expression was directly correlated with tumor growth. Moreover, when NSCLC tumor-bearing animals were depleted of ENA-78, both tumor growth and spontaneous metastases were markedly attenuated [78]. The reduction angiogenesis is also accompanied by an increase in tumor cell apoptosis, consistent with the previous observation that inhibition of tumor-derived angiogenesis is associated with increased tumor cell apoptosis [79, 80]. Similarly, *in vivo* and *in vitro* proliferation of NSCLC cells was unaffected by the presence of ENA-78. Although a significant correlation of ENA-78 exists with tumor-derived angiogenesis, tumor growth, and metastases, ENA-78 depletion does not completely inhibit tumor growth. This reflects that the angiogenic activity of NSCLC tumors is related to many overlapping and potentially redundant factors acting in a parallel or serial manner.

Prostate cancer tumorigenesis and metastasis is dependent on angiogenesis [81, 82]. Serum levels of IL-8 have been found to be markedly elevated in patients with prostate cancer. These levels are highly correlated with the stage of the disease and have been determined to be an independent variable from the ratio of free/total prostate specific antigen (PSA) [83]. In fact, the combined use of free/total PSA and IL-8 levels were more effective in distinguishing prostate cancer from benign prostatic hypertrophy. This suggests that an ELR$^+$ CXC chemokine may be playing an important role in mediating prostate cancer-derived angiogenesis in support of tumorigenesis and metastases. This observation in patients has been substantiated in human/SCID mice chimeras of human prostate cancer tumorigenesis [84]. Three human prostate cancer cell lines were examined for constitutive production of angiogenic ELR$^+$ CXC chemokines [84]. Tumorigenesis of the human prostate cancer cell line, PC-3, was shown to be attributable, in part, to the production of the angiogenic CXC chemokine, IL-8. Depletion of endogenous IL-8 inhibited PC-3 tumor growth in SCID mice, that was entirely attributable to inhibition of PC-3 tumor-derived angiogenesis. In contrast, the human prostate cancer cell line, Du145, was found to utilize a different angiogenic CXC chemokine, GRO-α, to mediate tumor-derived angiogenesis. Depletion of endogenous GRO-α, but not anti-IL-8, reduced tumor growth that was directly related to attenuated angiogenic activity. Thus, prostate cancer cell lines can utilize distinct CXC chemokines to mediate their tumorigenic potential. Similar findings have been shown in gastric carcinoma [85, 86]. The findings for the redundancy of ELR$^+$ CXC chemokines in human tumors provides the unique opportunity to target a putative receptor for ELR$^+$ CXC chemokine-mediated angiogenesis.

ELR$^-$ CXC chemokines attenuate angiogenesis associated with tumorigenesis

ELR$^-$ CXC chemokines have been shown to inhibit angiogenesis in several model systems. For example, Burkitt's lymphoma cell lines form tumors in nude mice [87]. Angiogenesis is essential for tumorigenesis of these lymphomas, analogous to carcinomas. The expression of IP-10 and MIG was found to be higher in tumors that demonstrated spontaneous regression, and was directly related to impaired angiogenesis [88]. To determine whether this effect was attributable to IP-10 or MIG, more virulent Burkitt's lymphoma cell lines were grown in nude mice and subjected to intra-tumor inoculation with either IP-10 or MIG. Both conditions resulted in marked reduction in tumor-associated angiogenesis [30, 89]. Although both IP-10 and MIG have been demonstrated to induce mononuclear cell recruitment via the interaction with their putative CXC chemokine receptor (CXCR3) [15–18, 44], the ability of both of these ELR$^-$ CXC chemokines to inhibit angiogenesis and induce lymphoma regression in nude mice support that these chemokines mediate their effects in a T cell-independent manner.

To examine the role of IP-10 in the regulation of angiogenesis in a carcinoma, the level of IP-10 from human surgical NSCLC tumor specimens was examined and found to be significantly higher in the tumor specimens than in normal adjacent lung tissue [90]. The increase in IP-10 from human NSCLC tissue was entirely attributable to the higher levels of IP-10 present in squamous cell carcinoma (SCCA) compared with adenocarcinoma. Moreover, depletion of IP-10 from SCCA surgical specimens resulted in augmented angiogenic activity [90]. The marked difference in the levels and bioactivity of IP-10 in SCCA and adenocarcinoma is clinically and pathophysiologically relevant, and represents a possible mechanism for the biological differences of these two cell types of NSCLC. Patient survival is lower, metastatic potential is higher, and evidence of angiogenesis is greater for adenocarcinoma, compared with SCCA of the lung [91–93]. These studies were extended to a SCID mouse system to examine the effect of IP-10 on human NSCLC cell line tumor growth in a T- and B cell-independent manner. SCID mice were inoculated with either adenocarcinoma or SCCA cell lines [90]. The production of IP-10 from adenocarcinoma and SCCA tumors was inversely correlated with tumor growth [90]. However, IP-10 levels were significantly higher in the SCCA, compared with adenocarci-

noma tumors. The appearance of spontaneous lung metastases in SCID mice bearing adenocarcinoma tumors occurred after IP-10 levels from either the primary tumor or plasma had reached a nadir. In subsequent experiments, SCID mice bearing SCCA tumors were treated with either neutralizing anti-IP-10 antibodies, whereas animals bearing adenocarcinoma tumors were treated with intra-tumor IP-10. Depletion of IP-10 in SCCA tumors resulted in a twofold increase in their size. In contrast, reconstitution of intra-tumor IP-10 in adenocarcinoma tumors reduced both their size and metastatic potential, which was unrelated to infiltrating neutrophils or mononuclear cells (i.e., macrophages or NK cells) and directly attributable to a reduction in tumor-associated angiogenesis.

The role of angiogenic (ELR^+) and angiostatic IFN-inducible (ELR^-) CXC chemokines in the regulation of angiogenesis associated with chronic fibroproliferative disorders

Angiogenesis is increasingly being recognized for its role in promoting the pathogenesis of chronic inflammatory/fibroproliferative disorders. For example, rheumatoid arthritis is associated with the unrestrained proliferation of fibroblasts and capillary blood vessels that leads to the formation of the pannus and destruction of joint spaces. Macrophages isolated from rheumatoid synovium produce pro-angiogenic factors [94]. Psoriasis is a well-known angiogenesis-dependent skin disorder that is characterized by marked dermal neovascularization. Keratinocytes isolated from psoriatic plaques demonstrate a greater production of angiogenic activity. It is interesting that this angiogenic phenotype is due, in part, to a combined defect in the overexpression of the angiogenic cytokine IL-8, and a deficiency in the production of the angiogenesis inhibitor, thrombospondin-1, resulting in a pro-angiogenic environment [95].

Idiopathic pulmonary fibrosis (IPF) is a chronic and often fatal pulmonary fibroproliferative disorder. The pathogenesis of IPF that ultimately leads to end-stage fibrosis demonstrates features of dysregulated/abnormal repair with exaggerated neovascularization/vascular remodeling, fibroproliferation, and deposition of extracellular matrix, leading to progressive fibrosis and loss of lung function. Although numerous eloquent studies have examined the biology of fibroblast proliferation and deposition of extracellular matrix (ECM) in interstitial lung disease, few studies have examined the role of angiogenesis/vascular remodeling that may support fibroplasia and deposition of ECM in these disorders.

The existence of neovascularization in IPF was originally identified by Turner-Warwick, who examined the lungs of patients with widespread interstitial fibrosis and demonstrated neovascularization leading to anastomoses between the systemic and pulmonary microvasculatures and evidence of extensive vascular remodeling in areas of fibrosis [96]. These findings have been further substantiated with evidence of extensive neovascularization during the pathogenesis of pulmonary fibrosis in bleomycin-induced pulmonary fibrosis [97].

Recently, studies have corroborated the findings of Turner-Warwick, and have shown that the bronchoalveolar lavage fluid and lung tissue from patients with IPF have marked angiogenic activity that is almost entirely attributable to the imbalance in the overexpression of the angiogenic ELR^+ CXC chemokine, IL-8, compared with the relative down-regulation of the angiostatic IFN-inducible CXC chemokine, IP-10 [98]. To determine whether the imbalance in the expression of these CXC chemokines were relevant to the pathogenesis of pulmonary fibrosis, studies were extended to a murine model system of bleomycin-induced pulmonary fibrosis. In this model system, the expression and biological activity of murine macrophage inflammatory protein-2 (MIP-2; an angiogenic ELR^+ CXC chemokine homologous to human GRO-β/γ) and the angiostatic CXC chemokine, IP-10, were correlated to the magnitude of lung fibrosis during bleomycin-induced pulmonary fibrosis [99, 100]. MIP-2 and IP-10 were measured during bleomycin-induced pulmonary fibrosis from bronchoalviolar lavage and whole lung tissue homogenates, and were found to be directly and inversely correlated, respectively, with total lung hydroxyproline levels, a measure of lung collagen deposition [99, 100]. Moreover, if either endogenous MIP-2 was depleted or exogenous IP-10 (intramuscular) was administered to the animals during bleomycin exposure, both treatment strategies resulted in marked attenuation of pulmonary fibrosis that was entirely attributable to a reduction in angiogenesis in the lung [99, 100]. These findings support the notion that angiogenesis is critical to promote fibroplasia and deposition of ECM during pulmonary fibrosis, and that angiogenic and angiostatic factors, such as ELR^+ and interferon-inducible ELR^- CXC chemokines play an important role in the pathogenesis of this process. Furthermore, with the recent demonstration of the efficacy of IFN-γ treatment of IPF patients [101, 102], the above studies substantiate that IFN-γ treatment of IPF may mediate its effect, in part, by shifting the imbalance of the expression of angiogenic ELR^+ and angiostatic interferon-inducible ELR^- CXC chemokines to favor an angiostatic environment leading to inhibition of dysregulated neovascularization/vascular remodeling, fibroproliferation, and deposition of extracellular matrix in IPF patients.

CONCLUSION

Angiogenesis is regulated by an opposing balance of angiogenic and angiostatic factors. CXC chemokines are a unique cytokine family that contains members that exhibit on a structural/functional basis either angiogenic or angiostatic biological activity. The above studies have demonstrated that, as a family, the CXC chemokines appear to be important in the regulation of angiogenesis associated with both tumorigenesis and the pathogenesis of chronic inflammatory/fibroproliferative disorders. These findings support the notion that therapy directed at either inhibition of angiogenic or augmentation of angiostatic CXC chemokines may be a novel approach in the treatment of solid tumors and chronic fibroproliferative disorders.

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Chemokine-mediated angiogenesis: an essential link in the evolution of airway fibrosis?

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Angiogenesis may be an important factor in the development of fibrotic lung disease. Prior studies have strongly suggested a role for angiogenic vascular remodeling in pulmonary fibrosis, and emerging evidence indicates that new vessel formation is critical in airway fibrosis. Bronchiolitis obliterans syndrome is a fibrotic occlusion of distal airways that is largely responsible for the morbidity and mortality of patients after lung transplantation. In this issue, Belperio et al. demonstrate a role for CXC chemokine receptor 2 in the regulation of angiogenesis-mediated airway fibroproliferation (see the related article beginning on page 1150). By integrating an understanding of neovascularization into the study of events that occur between inflammation and fibrosis, it becomes increasingly possible to rationally design therapies that can halt conditions of maladaptive fibrosis.

Neovascularization is an important component of fibrotic responses (1). In this issue of the *JCI*, Belperio and colleagues extend this relationship to the development of chronic lung transplant rejection (2). Using bronchoalveolar lavage fluid from patients with pending or established bronchiolitis obliterans syndrome (BOS) and tracheal allograft tissue from a mouse model of obliterative airway disease, the authors make a convincing case for the central role of CXC chemokine receptor 2 (CXCR2) regulation of angiogenesis-mediated airway fibroproliferation.

Airway inflammation and fibrosis in the evolution of BOS

Chronic allograft rejection is the chief factor limiting long-term survival following lung transplantation. BOS is the pathological correlate of chronic rejection and primarily affects the respiratory and terminal bronchioles, which culminates in a fibrotic occlusion of the distal airways (3). The cumulative incidence of BOS at 5 years after lung transplant is between 50% and 80%, and 5-year survival after BOS onset is only 30–50%. The International Society of Heart and Lung Transplantation Registry has noted that the development of BOS within the first year after transplantation is the single most important factor influencing 5-year mortality among patients undergoing lung transplantation (3). As a fibrotic disease, BOS is poorly responsive to standard immunosuppression employed by transplant physicians. Similarly, pulmonary fibrosis, which affects the lung interstitium rather than the conducting airways, responds poorly to immunotherapy and has long been associated with pathologic angiogenesis (4).

It is a generally recognized phenomenon that inflammation is an initiating event that precedes the progression to fibrosis in several lung diseases, including BOS and idiopathic pulmonary fibrosis. While fibrosis may be a frequent sequel of an acute or subacute inflammatory event, it is also clear that inflammation does not always result in fibrosis. The long-term effect of interstitial or airway fibrosis is irreversible lung architectural remodeling. Key questions regarding the mechanisms of airway remodeling are: (a) What are the specific inflammatory initiators? and (b) What is the sequence of events that culminates in fibroproliferation? In lung transplantation, the answer to the first question most certainly involves the response to alloantigen triggering of innate and adaptive immune responses. The answer to the second question is probably less well understood but is perhaps of greater importance in the development of therapies that reach beyond immunosuppression. Lung transplant clinicians well appreciate that acute rejection treated early may respond excellently to immunosuppressive therapies but that late intervention is rarely successful. Unfortunately, it is not always possible to intervene early, and occasionally even apparently early intervention with high-dose steroids or T cell–depleting strategies cannot halt the decline in lung function once fibroproliferation is initiated.

The potential role of CXC chemokines in angiofibroproliferative BOS

The study by Belperio and colleagues (2) firmly establishes that neovascularization is an important contributor to the process of fibroproliferation in airway fibrosis. The investigators present a cohesive and clearly argued interpretation of experimental data from human BOS patients and a well-characterized murine model of tracheal transplant rejection. Their findings make a convincing case for the central role of CXCR2-dependent Glu-Leu-Arg-positive (ELR$^+$) chemokine regulation of angiogenesis-mediated BOS fibroproliferation. The study extends their previ-

Nonstandard abbreviations used: BOS, bronchiolitis obliterans syndrome; CXCL, CXC chemokine ligand; CXCR2, CXC chemokine receptor 2.

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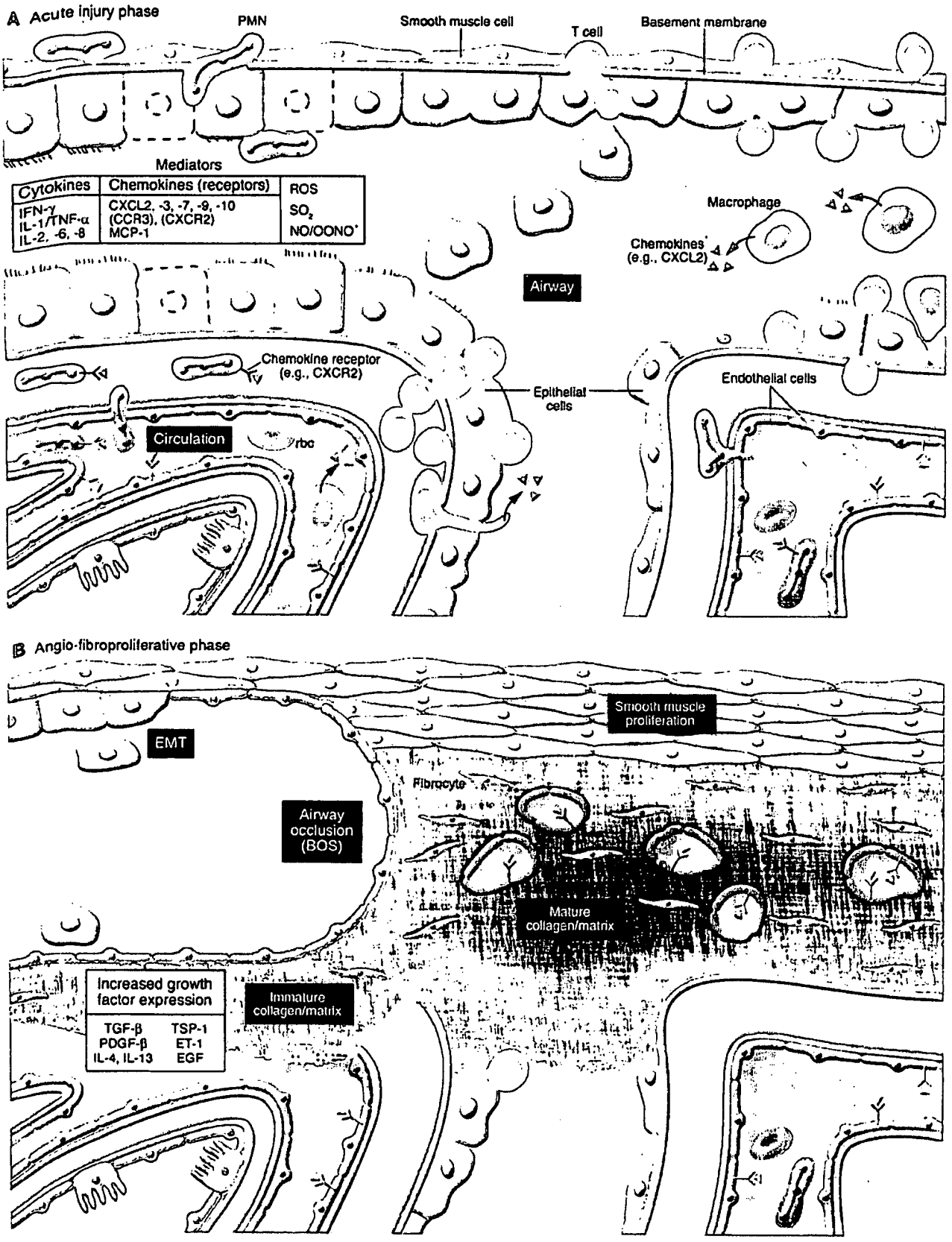
Exhibit 2



ously published observations based on the BOS model, which implicated monocyte chemoattractant protein-1/CXCR2-mediated mononuclear phagocytic infiltration in the progression of BOS (5). CXC chemokines, 1 of the 4 subfamilies of multifunctional growth- and immune-mediating molecules, regulate acute inflammatory responses and vascular remodeling. CXC chemokines contain an ELR$^+$ motif immediately before the first cysteine residue at the NH_2 terminus. This group includes CXC ligand 1 (CXCL1), -2, -3, -5, -6, and -8. Angiogenesis (both physiological and pathological) is potently stimulated by engagement of these ligands with G protein-coupled CXCR2 on endothelial cells. By contrast, IFN-regulated ELR$^-$ chemokines such as CXCL4, -9, -10, and -11 function as angiostatic regulators through CXCR3 and direct interaction with angiogenic factors such as VEGF (6).

The elucidation of VEGF-independent, chemokine-mediated angiogenesis is pivotal to our evolving understanding of the cascade of remodeling events that results in inexorable progression of fibroproliferative BOS. The present study also demonstrates a temporal separation of signaling responses to CXCR2 activation. CXCR2 engagement in early murine tracheal allograft BOS results in neutrophil trafficking, but at 3 weeks results in neutrophil-independent angiofibroproliferation (2). Moreover, treatment of CXCR2$^{-/-}$ mice with threshold doses of cyclosporin, insufficient to prevent BOS in wild-type mice but sufficient to inhibit early monocyte infiltration, resulted in a dramatic reduction in allograft fibroobliteration, which points to a clinically relevant strategy for further investigation.

How does pathological angiogenesis fit into what is already known or generally posited for lung fibrosis? Briefly, there is evidence to suggest that the epithelium can be an early target as well as a source of alloantigen (7). Following exposure to a new antigen or toxin, there is an influx of neutrophils and mononuclear cells (Figure 1A). This period of cellular infiltration is associated with an increased production of chemokines and cytokines. In this cascade, multiple changes occur in the epithelia, including apoptosis, hyperplasia, dedifferentiation, and metaplasia (8–12). Epithelial changes may stimulate underlying myofibroblasts in a paracrine fashion, with resultant collagen matrix deposition (13–15). In the midst of these changes, neovascularization is observed (Figure 1B). Perhaps as a consequence of increased vascularity and chemoattractant gradients, migratory cell populations (e.g., fibrocytes; ref. 16) traffic to the site of inflammation, possibly as part of a tissue repair response. As this process progresses, the cellular infiltrates diminish (8, 9), and airway remodeling proceeds in a unidirectional fashion, with extensive subepithelial, luminal, and/or interstitial fibrosis. Our group has recently established using an orthotopic (in situ) mouse tracheal transplant model that it is possible to reverse alloimmune injury that remains unmitigated for 7 days, but after 10 days of unprotected immune airway injury, fibroproliferative diseases cannot be ameliorated (8). Thus, it appears that a temporal sequence of events leading from inflammation to fibrosis may be closer to being fully elucidated.

In addition to alloimmune injury, several important potential amplifiers of BOS have been identified. These include chronic gastroesophageal reflux (17), community-acquired viral infection (18), and CMV infection (19), all of which are also associated with chronic graft rejection. Chemokine-mediated inflammation and angiogenesis may be a common pathway for progression to fibroproliferative BOS in response to superimposition of these factors on an immune-injured airway. For example, virulent CMV strains produce a potent ELR$^+$ chemokine analog, UL146 (also known as viral CXCL1). This CXCR2-restricted ligand is capable of inducing neutrophil chemotaxis independent of monocyte activation (20) and could potentially contribute to chemokine-mediated perigraft angiogenesis and fibroproliferation.

Belperio and colleagues' important observations (2) hold great promise for translational application in the therapy of BOS and other fibrotic lung diseases that involve pathological angiofibroproliferation. Presently, prevention of graft rejection requires intensive, prolonged immunosuppression with corticosteroids, calcineurin antagonists, antimetabolites, and immune modulators (21). This approach, which is focused on the inflammatory component, has clear limitations, with significantly increased risks of opportunistic infection and a limited effect on angiogenesis. An obvious therapeutic strategy would be treatment with a combination of lower doses of traditional immunosuppressants and humanized blocking antibodies against angiogenic chemokines or their receptors, as modeled in the present study (2). If this broad framework holds true for most fibrotic conditions, in the future, it may be possible to more appropriately treat other pulmonary, renal, dermatological, and cardiac diseases in which the inflammation-angiogenesis-fibroproliferative pathways are active.

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Figure 1

Evolution of BOS from airway injury to angiofibroproliferation. (**A**) The progression of airway pathology from initial injury to luminal and subepithelial fibrosis begins with cellular infiltration consisting of macrophages (a significant source of CXCL2), neutrophils, and subsequently T lymphocytes. Infiltrating cells and injured lung parenchyma (e.g., epithelial cells, vascular endothelial cells) liberate a variety of cytokines, oxidants, and chemokines. One prominently expressed chemokine, CXCL2, binds to receptors on neutrophils and vascular endothelium, simultaneously promoting a neutrophil influx and angiogenesis. Following cellular infiltration, the respiratory epithelium is likely injured, and pseudostratified columnar epithelium undergoes apoptosis and is transformed into a flattened, dysplastic monolayer that covers the basement membrane. (**B**) During a period when the epithelium is undergoing progressive injury, angiogenesis likely occurs through a CXCR2-dependent, VEGF-independent process (2). While the mechanism by which angiogenesis contributes to progressive airway fibrosis has not yet been fully elucidated, it is possible that increased vascularity facilitates the delivery of growth factors that promote tissue remodeling (e.g., platelet-derived growth factor-2 [PDGF2]) or cells that differentiate to produce collagen (e.g., fibrocytes). The concurrent and persistent immune epithelial injury may result in epithelial-myofibroblast crosstalk that promotes new collagen synthesis (22) through paracrine growth factor signaling and epithelial-to-mesenchymal transition (EMT). As subepithelial fibrosis increases and the respiratory epithelium becomes less distinct, increased deposition in the airway lumen of type I and III collagen occurs. Eventually, this fibrosis culminates as BOS, a physiologically significant occlusion of the airways in lung transplant recipients, which is the hallmark of chronic lung transplant rejection. PMN, polymorphonuclear neutrophil; ET-1, endothelin-1; EGF, epidermal growth factor.

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The role of hepatic insulin receptors in the regulation of glucose production

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The inability of insulin to suppress hepatic glucose production (HGP) is a key defect found in type 2 diabetes. Insulin inhibits HGP through both direct and indirect means, the latter of which include inhibition of glucagon secretion, reduction in plasma nonesterified fatty acid level, decrease in the load of gluconeogenic substrates reaching the liver, and change in neural signaling to the liver. Two studies in this issue of the *JCI* demonstrate that selective changes in the expression of insulin receptors in mouse liver do not have a detectable effect on the ability of insulin to inhibit HGP (see the related articles beginning on pages 1306 and 1314). These provocative data suggest that the indirect effects of insulin on the liver are the primary determinant of HGP in mice.

Introduction

Until late 1987, it was believed that insulin's ability to reduce hepatic glucose production (HGP) resulted from the direct interaction of the hormone with its receptor in the hepatocyte plasma membrane. This belief was called into question when Prager et al. (1) noted that in obese, nondiabetic humans, suppression of glucose production could occur in response to insulin infusion, even when the estimated portal vein insulin concentration did not rise. These results suggested that insulin also reduces hepatic glucose output by indirect mechanisms. Subsequent work by others supported this concept (2–5), and it is now recognized that insulin can inhibit HGP by both direct and indirect means (Figure 1).

Indirect actions of insulin on the liver

The indirect actions of insulin on HGP are diverse. Glucagon secretion from the α cell of the pancreas is diminished by insulin, which in turn causes a reduction in HGP (6). Likewise, nonesterified fatty acid (NEFA) release from the adipocyte is reduced by insulin, and a reduction in the supply of NEFAs to the liver causes an increase in hepatic glycolytic flux, resulting in glucose-6-phosphate exiting the liver after being converted to lactate rather than glucose (3). Additionally, the effect of insulin on fat and muscle reduces the supply of gluconeogenic precursors reaching the liver, again reducing HGP (2, 7). More recently, insulin's action in the brain has been postulated to play a role in the regulation of HGP (8). It is known that the brain can sense the circulating insulin level (9) and that it provides neural input to the liver (10). Further, it has been shown that infusion of insulin into the third ventricle of rats can reduce glucose production (8). Likewise, blocking insulin action in the rat hypothalamus impairs the ability of a physiologic rise in circulating insulin to inhibit HGP (8).

Direct actions of insulin on the liver

The exploration of insulin's indirect effects on the liver called into question the physiologic relevance of the hormone's direct hepatic effect, even though numerous in vitro studies had shown it to exist. Perhaps the best in vivo demonstration of the hormone's direct effect on the liver comes from studies conducted in the conscious overnight-fasted dog, in which changes in plasma insulin were brought about selectively in the liver using the pancreatic clamp technique.

Nonstandard abbreviations used: HGP, hepatic glucose production; LIRKO, liver insulin receptor knockout; NEFA: nonesterified fatty acid; NHGO, net hepatic glucose output.

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